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Dixie R. [signature]
Attorney for Applicant

12/6/96
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application Of	:	Group Art Unit 1804
LOUIS D. FALO ET AL.	:	Examiner J. Schmuck
Serial No. 08/535,556	:	Attorney Docket No. 125350
Filed September 28, 1995	:	
Entitled	:	
STIMULATION OF CELL-MEDIATED IMMUNE RESPONSES BY TARGETED PARTICULATE GENETIC IMMUNIZATION	:	

DECLARATION OF LOUIS D. FALO, JR.

I, LOUIS D. FALO, JR., being duly sworn hereby declare as follows:

1. I am a co-inventor of the invention set forth in the claims of the above-captioned application.

2. I received a B.S. Degree in Biochemistry/Chemistry from the University of Pittsburgh (1981); and an M.D./Ph.D. in Immunology from Harvard Medical School and Harvard University (1988).

3. I have held the following post graduate positions: Research Associate (Immunology), Department of Pathology, Harvard Medical School (1987-1988); Resident-PGY1, Department of Medicine, Massachusetts General (1988-1989); Resident, Department of Dermatology, Harvard Medical School (1989-1992); and Research Fellow, Division-Lymphocyte Biology, Dana-Farber Cancer Institute (1990-1992).

4. I am currently the Assistant Professor and Vice Chairman in the Department of Dermatology, University of Pittsburgh School of Medicine, a position that I have held since October 1996. Prior to that time, I was the Assistant Professor, Director of Research and Academic Affairs in the Department of Dermatology, University of Pittsburgh School of Medicine (October 1993 to October 1996), and an instructor in the Department of Dermatology, Harvard Medical School (1992-1993).

5. I have received the following honors and awards: Summa cum laude, B.S., University of Pittsburgh (1981); Senior of the Year, University of Pittsburgh (1981); Phi Beta Kappa (1981); Wellcome Cancer Research Award (1992); and Leadership Award - American Dermatology Association (1996).

6. I have authored or co-authored the publications listed in Exhibit I.

7. I am a co-author of the publication attached as Exhibit II.

8. I am a member of the following professional societies: American Association for the Advancement of Science (1993 to Present); Society of Investigative Dermatology (1992 to Present); American Assocation of Immunologists (1995 to Present); and the American Association of Cancer Research (1995 to Present).

9. I participated in the preparation of the above-captioned application and claims, read the same thoroughly before the case was filed, and I have recently re-read and reviewed the application and claims of the case.

10. I have carefully read the Office Action dated August 6, 1996 which included an objection to the specification under 35 U.S.C. § 112, first paragraph, for failing to provide an enabling disclosure and a rejection of Claims 1-67 on the same basis.

11. In addition to the experimental data provided in the application, under my direction and control, the following experiments were performed. These experiments utilized the technology taught in the patent application.

12. Genetic immunization via biolistic administration as discussed in Example Section 7 of the application was repeated. More specifically, DNA coated gold particles were prepared as discussed in the specification on page 25, lines 10-20. Naive C57BL/6 mice were immunized with a total of 2.00 micrograms of OVA encoding DNA delivered to the abdominal skin with two spatially overlapping pulses, and boosted in an identical fashion 7 days later. Control mice were immunized with DNA encoding the irrelevant antigen β-galactosidase. Both groups of mice were challenged 7 days following immunization by intradermal injection of the MO4 or B16 melanoma at a distant site. All of these steps were performed according to the methodology detailed in Example Section 7. This confirmed that ova-immunized mice were protected from the lethal tumor challenge, while tumors in control mice continued to grow and were uniformly lethal by day 34 following injection. In addition, OVA-immunized mice were not protected from challenge with the untransfected parent melanoma B16, indicating that protective immunity was antigen specific depending on OVA expression by the tumor target. Based upon these results, I would conclude that the treatment given to mice according to the methods disclosed and claimed in the pending application resulted in a prophylactic vaccine against tumor growth.

12. Further testing was performed to investigate the mechanism of immunization in this mouse model. Naive C57BL/6 mice were immunized with 2.00 micrograms of plasmid DNA encoding the Lantern variant of the reporter gene for green fluorescent protein (GFP) in the same manner described in Example Section 7. This gene encodes a naturally fluorescent protein requiring no substrate for visualization. DNA coated particulates were constructed and delivered by the same techniques used for OVA immunization. Draining lymph nodes of immunized mice were excised and sectioned 24 hours after immunization. By use of electron microscopic analysis, it was determined that cells within the lymph nodes that had dendritic cell form and structure appeared to contain 1 micrometer electron dense particles, and serial sections demonstrated intracytoplasmic locale of the particles. Cytospins of single-cell suspensions from the draining lymph nodes were examined by fluorescence microscopy to confirm expression of the delivered GFP genes. Green fluorescence in cells from the GFP immunized mice indicated selective expression of GFP in cells with dendritic cell form and structure. In addition, gold particles could be

detected in these cells under bright field microscopy. Based upon these results, I would conclude that particle containing dendritic cells in the draining lymph nodes were expressing the genetically introduced protein. Thus, the methods of the present invention are effective in delivering a DNA encoding protein to APCs.

14. Further studies were performed to determine whether the transfected dendritic cells discussed in Paragraph 13 were derived from the skin. Shaved abdomens of naive mice were painted with rhodamine immediately before biolistic immunization with GFP described in Paragraph 13. This rhodamine painting serves to label the cutaneous dendritic cells and facilitates analysis of cutaneous dendritic cell trafficking to the regional lymph nodes. Twenty-four hours after immunization, draining lymph nodes were excised and sectioned for analysis. Clusters of skin-derived cells (red fluorescence) were evident within the lymph nodes in the region of afferent lymph flow. Several cells contained both the red fluorescence from the rhodamine and green fluorescence from GFP expression. These results clearly demonstrate that the GFP expression was the result of skin-derived dendritic cells within the draining lymph node, as taught in the present invention.

15. The results discussed in Paragraphs 12, 13 and 14 are further discussed in the article attached as Exhibit II.

16. By following the procedures detailed in the patent application and as defined by the pending claims, I would conclude that the disclosed invention teaches that genetic immunization induces potent antigen-specific CTL responses and antigen-specific, CTL-dependent protective tumor immunity; and that transfected dendritic cells from the skin will migrate to the lymph nodes, where DNA encoding for a protein is expressed. It is my well-considered opinion that anyone skilled in the art could repeat the methods disclosed in the pending application without undue experimentation to achieve these results. Therefore, after reviewing this Office Action and the specification, and in light of the more recent results discussed above, it is my further well-considered opinion that the specification is clearly enabling to one skilled in the art and that Claims 1-67 could be practiced without undue experimentation by such an individual.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further

that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

12/5/96
Date

Louis D. Falo, Jr.

EXHIBIT I

PUBLICATIONS
(16 of 21)

1. **Falo LD Jr.**, Sullivan K, Benacerraf B, Mescher MF, and Rock KL. Analysis of antigen-presentation by metabolically inactive accessory cells and their isolated membranes. *Proc. Natl. Acad. Sci. USA.* 1988,82: 6647-6651.

2. **Falo LD Jr.**, Benacerraf B, and Rock KL. Phospholipase pretreatment of antigen pulsed accessory cells selectively inhibits antigen-specific MHC restricted, but not allospecific stimulation of T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 1986, 83: 6694-6997.

3. **Falo LD Jr.**, Haber SI, Herrmann S, Benacerraf B, and Rock KL. Characterization of antigen association with accessory cells. I. Specific removal of processed antigens from the cell surface by phospholipases. *Proc. Natl. Acad. Sci. USA.* 1987, 84: 522-526.

4. **Falo LD Jr.**, Benacerraf B, Rothstein L, and Rock KL. Cerulenin is a potent inhibitor of antigen processing by antigen-presenting cells. *J. Immunol.* 1987, 139: 3918-3923.

5. Williams WW, **Falo LD Jr.**, Lu CY, Bennacerraf B, and Sy MS. Effects of in vitro monoclonal anti-I-A antibody treatment in neonatal mice of intrathymic and peripheral Class II antigen expression. *Cell. Immunol.* 1988, 111: 126-138.

6. **Falo LD Jr.**, Colarusso, LJ, Benacerraf B, and Rock KL. Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA.* 1992, 89: 8347-8350.

7. Razi-Wolf Z, **Falo LD Jr.**, and Reiser, H. Expression and function of the costimulatory molecule B7 on murine langerhans cells. *Eur. J. Immun.* 24:805-811, 1994.

8. **Falo LD Jr.**, Sober AJ, and Barnhill, RL. Evolution of Nevus Spilus. *Dermatology.* 189:382, 1994.

9. **Falo LD Jr.**, Kovacsovics-Bankowski, M., Thompson, K. and Rock, K.L. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumor immunity. *Nature Medicine.*1:649-653, 1995.

10. Mayordomo, J.I., Zorina, T., Storkus, W.J., Celluzi, C.M., **Falo LD, Jr.**, Ildstad, S.T., Kast, M.W., DeLeo, A.B., and Lotze, M.T. Bone marrow-derived dendritic cells pulsed with tumor peptides effectively treat established murine tumors. *Nature Medicine* 1:1297-1302, 1995.

11. Kress, DW, Seraly, MP, **Falo, LD, Jr.**, Kim B., Jegasothy, BJ, and Cohen B. Olmstead Syndrome. *Arch Dermatol.* 1996. 132:797-800.

12. Gonzalex S., vibhagool C, Falo, LD, Momtaz KT, Grevlink J, Gonzalez E. Treatment of pyogenic granuloma with the 585 NM pulsed dye laser. J. Am. Acad. Dermatology. 35:428-431. 1996.

13. Celluzzi, C., J.I. Mayordomo, W. J. Storkus, M.T. Lotze, and **Falo, LD, Jr.** Peptide-pulsed dendritic cells induce antigen specific CTL-mediated protective tumor immunity. *J. Exp. Med.* 183:283-287, 1996 .

14. Condon, C, S. Watkins, Celluzzi, C., Thompson, K, and **Falo, LD, Jr.** DNA-based Immunization by *in vivo* transfection of dendritic cells. *Nature Medicine* 2(10) 1122-1128. 1996

15. C. Celluzzi and **Falo, LD, Jr.** Epidermal dendritic cells induce antigen-specific CTLs-mediated immunity Submitted.

16. **LD Falo Jr.**, and Lotze MT. Cancer Vaccines. In Methods in Clinical Immunology. eds T Whiteside et al. Saunders. In press.

EXHIBIT II

Exhibit II
of Paper 11

DNA-based immunization by *in vivo* transfection of dendritic cells

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Delivery of antigen in a manner that induces effective, antigen-specific immunity is a critical challenge in vaccine design. Optimal antigen presentation is mediated by professional antigen-presenting cells (APCs) capable of taking up, processing and presenting antigen to T cells in the context of costimulatory signals required for T-cell activation. Developing immunization strategies to optimize antigen presentation by dendritic cells, the most potent APCs, is a rational approach to vaccine design. Here we show that cutaneous genetic immunization with naked DNA results in potent, antigen-specific, cytotoxic T lymphocyte-mediated protective tumor immunity. This method of immunization results in the transfection of skin-derived dendritic cells, which localize in the draining lymph nodes. These observations provide a basis for further development of DNA-based vaccines and demonstrate the feasibility of genetically engineering dendritic cells *in vivo*.

The goal of vaccination is to induce antigen-specific immunity to protect the host. Current vaccines can elicit effective antibody responses. However, the induction of cytotoxic T-lymphocyte (CTL) responses has been problematic. CTLs are an important component of the immune response to virally infected cells and tumors. CTLs kill neoplastic cells through the recognition of antigenic peptides presented by MHC class I molecules on the surface of the tumor target[1]. These peptides are derived from tumor antigens that are synthesized by the affected cell and degraded in the cytosol of the tumor target[2]. Although the recognition of peptide–class I complexes is sufficient to trigger target cell lysis, priming of CTL responses requires the presentation of the relevant antigen by professional antigen-presenting cells (APCs) capable of providing costimulation. Attempts to induce tumor-specific CTL responses *in vivo* by immunization with killed tumor cells or component proteins have generally been unsuccessful, presumably because proteins in the extracellular fluids cannot enter the cytosol and access the MHC class I presentation pathway.

Genetic immunization has been shown to induce humoral and CTL-mediated immune responses *in vivo*[3–9]. Through genetic immunization, the gene encoding a target antigen can be introduced into the cytoplasm of a cell, resulting in endogenous production of the antigen and presumably MHC class I access. Several *in vivo* gene transfer methods result in significant transgene expression, including retroviral or adenoviral mediated gene transfer and the direct injection of naked DNA.

The mechanism of genetic immunization is unknown[10]. Humoral responses may be explained by the secretion of antigen from transfected somatic cells, or by release of antigen as a result of cell lysis. Exogenous proteins released in this fashion could be taken up and presented to $CD4^+$ T cells by APCs in the draining lymph nodes.

The mechanism of presentation of genetically introduced antigens to $CD8^+$ CTLs is less clear. One possible scenario is that transfected nonprofessional APCs could present the immunogen directly, as transfection would result in endogenous synthesis and MHC class I access. However, this model does not take into account the absence of costimulation, typically provided by professional APCs. Another possibility is that lysis of transfected cells results in the uptake and presentation of antigens associated with cellular debris via phagocytosis by professional APCs. Phagocytosis of antigens allows access to the MHC class I pathway in professional APCs and induction of antigen-specific, CTL-mediated tumor immunity[11,12]. Similarly, it is possible that heat shock proteins could deliver peptides derived from antigens expressed in transfected cells to the MHC class I-restricted presentation pathway of professional APCs (ref. 13). Finally, it is possible that the actual process of genetic immunization directly transfects professional APCs. This would result in endogenous production of antigen within professional APCs and class I presentation in the context of appropriate costimulation for T-cell activation. Though genetic immunization can result in antigen expression by a variety of somatic cells (including myocytes[14] and keratinocytes[15]), the *in vivo* transfection of professional APCs has not been described.

Biolistic delivery of naked DNA is a physical method of DNA delivery, facilitating controlled delivery of DNA without the use of viral vectors[16]. Using gene gun technology, a single cutaneous immunization with as little as 1 μg of DNA can result in significant levels of transgene expression[16]. Because the skin is rich in dendritic cells, we hypothesized that cutaneous bombardment with DNA using the gene gun would result in direct transfection of these cutaneous dendritic cells. In this study we sought to determine the capacity of cutaneous biolistic genetic immunization to elicit protective immunity to lethal tumor challenge and to investigate the mechanism of this form of genetic immunization.

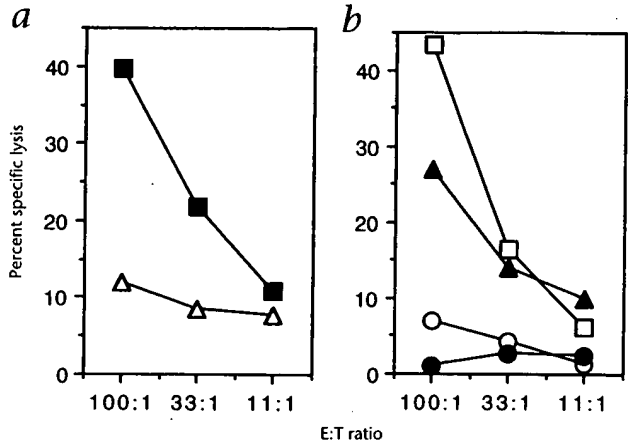

Fig. 1 Immunization by cutaneous delivery of OVA encoding DNA induces OVA-specific CTLs. *a*, *In vitro* restimulated splenocytes from OVA-immunized mice were assayed for cytolytic function against the OVA-transfected lymphoma EG7 (■) or the untransfected parent EL4 (△). *b*, Effector populations were incubated with complement alone (□) or with mAbs against CD4⁺ (▲), CD8⁺ (○), or Thy-1.2⁺ (●) lymphocytes and complement, then assayed for cytolytic activity against EG7 targets. Results are reported as the means of triplicate cultures for the effector:target ratios listed. The s.e.m. of triplicate cultures was always less than 15% of the mean. Experiments were repeated three times with similar results.

Induction of tumor immunity

To determine the potential of genetic immunization to induce protective tumor immunity, we utilized a tumor model consisting of the poorly immunogenic C57BL/6 mouse-derived melanoma B16 (ref. 17), and a subclone engineered to express the foreign antigen protein ovalbumin (OVA)[12]. Foreign antigen genes transfected into tumor cells behave like tumor antigens[12,18-22]. In this model, OVA functions as a defined model tumor antigen. The OVA-transfected B16 subclone MO4 endogenously synthesizes OVA and generates and presents the OVA peptide SIINFEKL with its surface class I molecule K^b (ref. 12). The expression of the OVA antigen does not significantly increase the immunogenicity of this tumor *in vivo*, as tumor growth and progression are similar to that of the untransfected parent[12]. This type of model has been used by numerous investigators to evaluate tumor-specific immunity[12,18-22].

In initial experiments, we evaluated the capacity of biolistic immunization to induce antigen specific CTLs. Naive C57BL/6 mice were immunized with a total of 2.00 μg of OVA encoding DNA delivered to the abdominal skin with two spatially overlapping pulses and boosted in an identical fashion 7 days later. *In vitro* restimulated spleen cells from these mice lysed the syngeneic OVA-expressing murine thymoma EG7, but not the untransfected parent tumor EL4 (Fig. 1*a*). Thus, target cell lysis was antigen specific and dependent on expression of OVA by the tumor target. Depletion of T-cell subsets from the effector populations using monoclonal antibodies demonstrated that lysis depended on Thy-1⁺, CD8⁺ subsets characteristic of MHC class I-restricted CTL effector cells (Fig. 1*b*).

In order to determine the capability of biolistic immunization in inducing protective tumor immunity, groups of mice that were immunized and boosted as described above were challenged 7 days later by intradermal injection of the MO4 or B16 melanoma at a distant site. OVA-immunized mice were protected from lethal tumor challenge, whereas tumors in control mice (immunized similarly, but with naked DNA encoding the irrelevant antigen β-galactosidase) continued to grow and were uniformly lethal by day 34 (Fig. 2*a*). OVA-immunized mice were not protected from challenge with the untransfected parent melanoma B16 (Fig. 2*b*), indicating that protective immunity was antigen specific, depending on OVA expression by the tumor target. We evaluated the contribution of CD8⁺ effector cells to this protective tumor immunity by depleting groups of immunized or control (*lacZ*-immunized) animals of CD8⁺ effector cells through repeated intraperitoneal (i.p.) injection of anti-CD8 monoclonal antibody before tumor challenge[12]. Although OVA-immunized animals were protected from MO4 challenge, survival in immunized, CD8⁺ T cell-depleted animals was similar to that observed in control animals, with or without T-cell depletion (Fig. 2, *c* and *d*). Therefore, CD8⁺ T cells are essential for the protective tumor immunity induced by genetic immunization in this model.

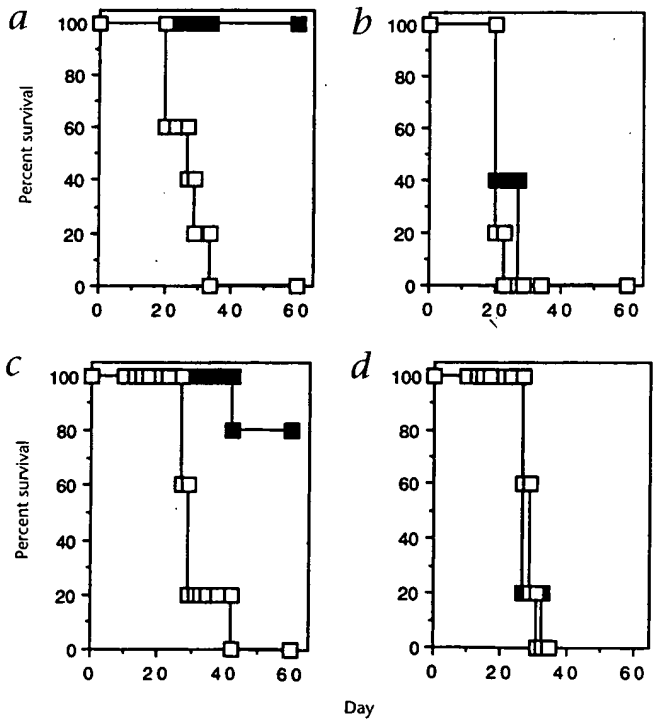

Fig. 2 Immunization by cutaneous delivery of OVA encoding DNA induces antigen-specific, CTL-mediated protection from lethal challenge with the OVA expressing melanoma MO4. Mice were genetically immunized with OVA (■) or *lacZ* (□) and boosted 7 days later. Groups of immunized mice were challenged 7 days after the final immunization (day 0) with either the B16 melanoma (*b*) or the OVA expressing subclone MO4 (*a*). Alternatively, immunized mice were divided into two groups, one of which was depleted of CD8⁺ lymphocytes by i.p. injection of anti-CD8 mAb 7 and 9 days after the last immunization. Intact (*c*) and CD8⁺ depleted (*d*) mice were then challenged 10 days after the final immunization (day 0) with MO4. Survival was reported as the percentage of surviving animals. Animals surviving on day 60 had no sign of tumor growth. All experiments included five mice per group and were repeated at least three times. Mice that became moribund were killed according to animal care guidelines.

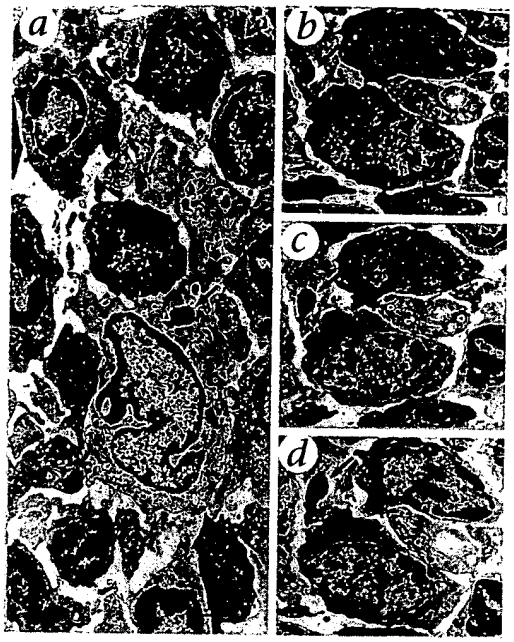

Fig. 3 Presence of particles in dendritic cells within lymph nodes draining the site of immunization. Mice were immunized as described in Fig. 1. Animals were killed 24 hours later, and draining lymph nodes were harvested and processed as described in the Methods section and observed using a JEOL 100CX microscope. *a*, A dendritic cell containing a single gold particle within the cytoplasm may be seen. Morphologic indices were used to confirm the identity of the cell; principally these are size, a paucity of cytoplasmic granules, and the presence of cytoplasmic veils. To confirm the cytoplasmic locale of the gold particles, serial sections were cut. It can be seen in *b–d* that the presence of the gold particles continues throughout all three sections (separated by 150 nm).

Transfection of dendritic cells *in vivo*

To investigate the mechanism of immunization in this model, we immunized naive C57BL/6 mice with a total of 2.00 μg of plasmid DNA encoding the Lantern variant of the reporter gene for green fluorescent protein (GFP). This gene encodes a naturally fluorescent protein requiring no substrate for visualization. DNA-coated particulates were constructed and delivered by the same techniques used for OVA immunization. Draining lymph nodes of immunized mice were excised and sectioned 24 hours after immunization. Electron microscopic analysis revealed cells within the lymph nodes with dendritic cell form and structure that appeared to contain 1-μm electron-dense particles (Fig. 3*a*). Serial sections demonstrated intracytoplasmic locale of the particles (Fig. 3, *b–d*). To confirm expression of the delivered GFP genes, cytospins of single-cell suspensions from draining lymph nodes excised 24 hours after immunization were examined by fluorescence microscopy. Green fluorescence in cells from GFP-immunized mice indicated selective expression of GFP (Fig. 4*a*) in cells with dendritic cell form and structure. Differential interference contrast imaging shows the positively staining cells to have a dendritic cell form and structure (Fig. 4*b*), consistent with the electron microscopic studies. Furthermore, gold particles could be detected in these cells quite easily under bright-field microscopy (Fig. 4, *b–d*). Lymph node cells from control mice, identically immunized with particles coated with irrelevant plasmid, contained particles but did not fluoresce (data not shown). Together, these results demonstrate expression of genetically introduced protein by particle-containing dendritic cells in the draining lymph nodes.

Transfected dendritic cells are derived from the skin

In order to determine the origin of the transfected dendritic cells, the shaved abdomens of naive mice were painted with rhodamine immediately before biolistic immunization with GFP. This treatment labels cutaneous dendritic cells and facilitates analysis of cutaneous dendritic cell trafficking to the regional lymph nodes[23,24]. Twenty-four hours after immunization, draining lymph nodes were excised and sectioned for analysis. Clusters of skin-derived cells (red fluorescence) were evident within the lymph nodes in the region of afferent lymph flow (Fig. 5*a*). Overlays of green fluorescence (GFP expression) revealed several double-positive cells, demonstrating GFP expression in skin-derived dendritic cells within the draining lymph node (Fig. 5*b*).

Discussion

Genetic immunization is an attractive approach for the induction of viral or tumor immunity. Vaccination with naked DNA can induce both humoral and cell-mediated immune responses and protection against viral infection or tumor challenge. Naked DNA-based vaccines offer several potential advantages over viral mediated transduction[9,10]. Among these is the potential for rapid and inexpensive production of large-scale DNA preparations. Such vaccine preparations can be prepared with relative purity and would be significantly more stable than current protein-based vaccines. Furthermore, the use of naked DNA vaccines would be inherently safer than viral mediated gene transfer, particularly in a potentially immunocompromised host. In addition, the use of naked DNA would eliminate immune responses to viral carriers, which can result in rapid elimination of transduced cells or limit the effectiveness of readministration.

Genetic immunization induces tumor-specific immunity. Several studies have shown that immunization with naked DNA against either natural or model tumor antigens can induce immunity to tumors expressing the antigen gene. A variety of strategies are being investigated to optimize this form of immunization. Recently, Conroy *et al.* have shown that predelivery of DNA encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) augmented carcinoembryonic antigen (CEA)-specific immunity induced by cutaneous particle bombardment with CEA encoding DNA (ref. 25). In another variation, Ciernik *et al.* have shown that biolistic genetic immunization with a minigene encoding a single epitope from mutant p53 targeted to the endoplasmic reticulum was partially able to protect mice from p53-expressing tumors[26]. Furthermore, Irvine *et al.* have shown regression of β-galactosidase-expressing tumors when gene gun immunization with the gene encoding β-galactosidase is carried out in combination with adjuvant cytokine therapy[27].

Current attempts to optimize naked-DNA immunization are proceeding without a clear understanding of the mechanism of this form of immunization. In this report, we address the mechanism of biolistic cutaneous genetic immunization. This method of naked-DNA immunization has been shown to be several times more efficient than other forms of inoculation[6]. We hypothesized that the effectiveness of this method may be in part due to the presence of significant numbers of dendritic cells in the tar-

get tissue. The skin is rich in dendritic cells, and these dendritic cells are capable of migrating to the regional lymph nodes where they exhibit potent APC function[23,24].

Our results demonstrate that biolistic genetic immunization induces potent antigen-specific CTL responses and antigen-specific, CTL-dependent protective tumor immunity. To show this we have used the nonimmunogenic murine melanoma B16 and a transfected subclone expressing the model tumor antigen OVA. The expression of the OVA antigen does not significantly increase the immunogenicity of this tumor *in vivo*, as tumor growth and protection are similar to that of the untransfected parent[12]. In our model OVA is endogenously synthesized by the transfected melanoma, and the SIINFEKL epitope is generated and presented with the class I molecule K^b on the surface of transfected cells[12]. In this respect, the nonself antigen OVA behaves as do many "natural" tumor antigens that are derived from viruses or mutated "altered-self" host proteins. It should be noted, however, that several identified tumor antigens are derived from nonmutated self antigens and that additional investigations will be needed to determine whether conclusions derived from nonself models are as applicable to such "autoantigens." Nevertheless, because the biology of the processing and presentation of antigen in this model is the same as that of many naturally occurring nonself tumor antigens, results from this model should be applicable to a wide variety of human tumors. The use of this well-defined model and others like it[18–21,27] is important for studies designed to define and optimize critical parameters for tumor immunization and will also facilitate direct comparisons between alternative immunization strategies[12,22].

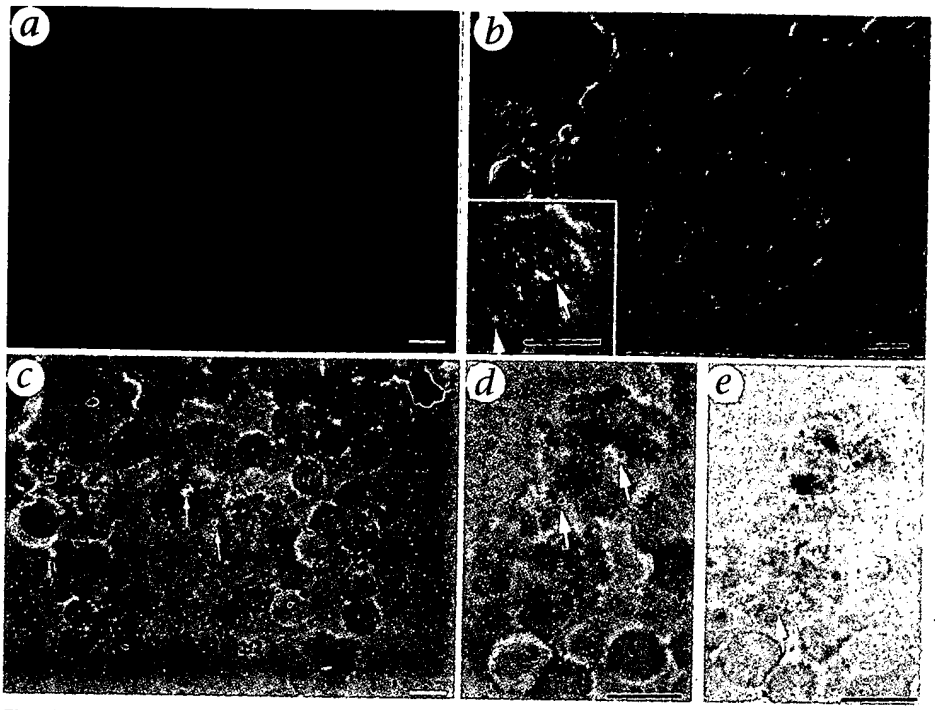

Fig. 4 Expression of introduced protein by genetically engineered, particle-containing dendritic cells in the draining lymph nodes. Mice were immunized as described in Fig. 1 except that particles were coated with the pGREEN LANTERN-1 plasmid (Gibco BRL), which contains the "humanized" reporter gene encoding GFP from the *Aequorea victoria* jellyfish. GFP is a naturally fluorescent protein. Draining lymph nodes were excised 24 h after immunization. Cytospins of lymph nodes were used to detect GFP-positive cells by fluorescence microscopy as described in Methods. Observation was with a Nikon photomicroscope using a 60X 1.4NA plan-apochromatic objective. *a*, The green fluorescent protein image is shown, signal from the GFP is clearly restricted to the cells central within the field (arrows). *b*, The differential interference contrast microscope image of the same fields shows the two cells to be dendritic (arrows), surrounded by smaller round lymphocytes. *b, inset*, At higher power gold particles may be visualized. *c*, The presence of the gold particles is more clearly detected using bright-field imaging (arrows). *d* and *e*, In fact, gold particles are detected in both GFP-positive cells at different focal planes (scale bar, 10 mm). Similarly prepared sections from naive animals do not demonstrate these distinctly colored, uniform beads.

Mechanism of biolistic genetic immunization. Our results show that biolistic cutaneous genetic immunization results in the presence of bombarded projectiles in the cytoplasm of dendritic cells in the draining lymph nodes. Identical delivery of DNA encoding the marker protein GFP demonstrates that introduced genes can be expressed as proteins by particle-containing dendritic cells in the lymph nodes. This is the first demonstration of *in vivo* transfection of dendritic cells that we are aware of. That these dendritic cells are skin-derived is demonstrated by the presence of double-stained skin-derived dendritic cells (red fluorescence) expressing GFP (green fluorescence) in tissue sections of the draining lymph node.

In combination, the data presented here support the hypothesis that biolistic immunization results in delivery of DNA to cutaneous dendritic cells. Thereafter, transfected dendritic cells migrate to draining lymph nodes, as they do for the presentation of protein and hapten antigens. Endogenously synthesized antigen can access the MHC class I-restricted pathway of transfected dendritic cells and can be presented to T cells in the lymphoid tissue with appropriate costimulatory signals for T-cell activation. Our studies do not rule out the possibility that DNA-coated beads may travel through the lymphatics to the lymph nodes where they may be directly captured by resident dendritic cells. In this regard, recent studies suggest that immature dendritic cells capable of particulate phagocytosis exist in the hepatic lymph and spleen[28], and our previous studies and others[11,12] suggest that subcutaneously administered particle-associated antigens can access the cytosol of phagocytic cells through a phagosome-to-cytosol pathway *in vivo*. It is unclear whether *in vivo* administered particle-bound naked DNA could survive trafficking to the lymph nodes, uptake by resident dendritic cells, and endosomal transport and escape and still be functionally expressed. We believe this would be unlikely in this system, as DNA coated onto gold beads by this method is rapidly solubilized in aqueous media with greater than 95% of the DNA dissociating from the beads in less than 3 min (data not shown). It is possible that such solubilized DNA may traffic to the regional lymph

nodes and be taken up and expressed by resident dendritic cells independently of the beads, but it is unlikely that this mechanism alone would account for the colocalization of gene expression and particles within cells, the transfection of skin-derived dendritic cells, and the high efficiency of transfection we observe.

In common with previous reports[14,15], we also observe antigen expression in nonprofessional APCs at the site of immunization (data not shown). It is possible that antigen released by these cells could indirectly be taken up by APCs and subsequently presented to T cells as well. Elegant studies by Huang *et al.* demonstrate that CTL induction by such "cross-priming" alone would require some mechanism for antigen access to the TAP-dependent classical MHC class I-restricted processing pathway[29]. The direct transfection of dendritic cells would explain the remarkable efficiency of this form of immunization.

It is unclear whether CTL induction by genetic immunization is helper cell dependent. These studies demonstrate that $CD8^+$ CTLs are the predominant effector cells in the protective tumor immunity we observe, but do not address the role of $CD4^+$ T cells in CTL induction. Dendritic cells have an exceptional ability to stimulate naive $CD4^+$ T cells, and several studies have shown that cutaneous biolistic genetic immunization elicits helper cell-dependent antibody responses[3,6,15], presumably by MHC class II-restricted presentation of exogenous antigen released from transfected cells[10]. Recent studies suggest that induction of CTLs by dendritic cells pulsed with MHC class I-restricted peptides requires the presentation of MHC class II-restricted epitopes and activation of $CD4^+$ T cells[30,31]. In contrast, studies by Ciernik *et al.* have shown induction of CTLs by gene gun immunization with DNA encoding only a class I-restricted T-cell epitope[26]. Although the OVA construct used in our studies includes $CD4^+$ T-cell epitopes, our experiments do not directly address whether CTL induction is helper cell dependent in this model.

Currently there is significant interest in the potential use of dendritic cells as adjuvants for immunization. Dendritic cells can be expanded *in vitro*, loaded with diverse forms of antigens including peptides, proteins, and antigen-encoding genes, and reinjected into the host[22,32-34]. Our results demonstrate that dendritic cells can be transfected *in vivo*, potentially obviating the need for *in vitro* expansion, manipulation and reinfusion. This finding also raises the possibility that dendritic cells could be engineered *in vivo*, through the genetic cointroduction of antigens and immunoregulatory molecules, to induce or suppress antigen-specific immune responses in the host.

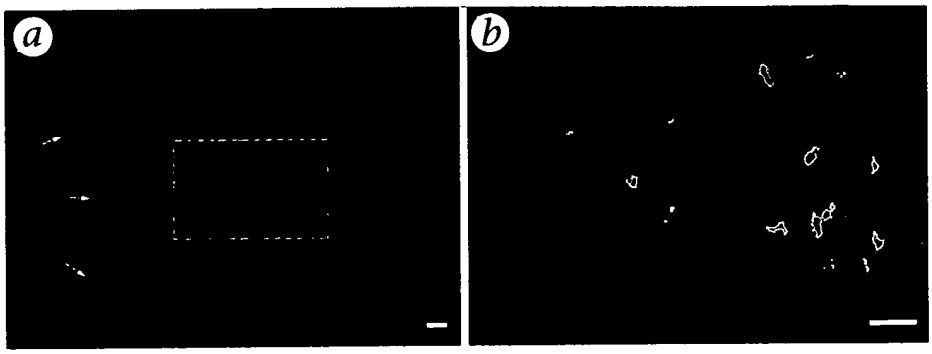

Fig. 5 Transfected lymph node dendritic cells are derived from immunized skin. To detect the presence of skin-derived dendritic cells and the presence of GFP within these cells, lymph nodes from mice painted with rhodamine immediately before immunization with GFP were harvested, fixed in 2% paraformaldehyde in PBS, cryoprotected in 30% sucrose, and shock frozen. Cryosections (5 μm) were cut through the belly of the node and immediately mounted and observed as described in the Methods. *a*, At low power, the skin-derived cells within the node are detected with a rhodamine cube set. It was notable that the majority of the cells occurred in large islands, generally located at the periphery of individual nodes [in this image the edge of the node is delineated (arrows)]. When the rhodamine signal (reflecting skin-derived dendritic cells) and the GFP signal (reflecting positive transfection and active production of GFP) are superimposed, it is clear that many of the red dendritic cell profiles also produce an abundant GFP signal, seen as a yellow signal in *b*. Occasional profiles that contain predominantly green signal are present in this image. This is considered to reflect cells that either were successfully transfected with GFP, although they were not stained by the topical rhodamine, or the cell section is at the limit of the cytoplasm such that some GFP signal occurs but none of the predominantly plasmalemmal rhodamine is detectable. Scale bar, 25 μm. Rhodamine signal is absent in unpainted animals.

Methods

Mice and cell lines. Female C57BL/6 mice, 5–8 weeks old, were purchased from the Jackson Laboratories (Bar Harbor, Maine). EL4 is a C57BL/6 T lymphoma, and EG7 is a chicken egg ovalbumin (OVA)-transfected subclone of EL4 (ref. 21, kindly provided by M. Bevan). The C57BL/6-derived murine melanoma B16 (ref. 17) was obtained from the American Type Culture Collection [(ATCC), Rockville, Maryland]. MO4 was constructed by transfection of B16 with the pAc-neo-OVA plasmid as described[12]. Monoclonal antibodies were prepared from the hybridomas GK1.5 (anti-CD4, ATCC TIB-207), 2.43 (anti-CD8, ATCC TIB-210) or 30-H12 (anti-Thy-1.2, ATCC TIB-107). Ascites containing anti-CD8 antibodies was raised in BALB/c *nu/nu* mice by i.p. injection of GK1.5 cells (3×10^6) and incomplete Freund's adjuvant (0.5 ml per mouse).

Genetic immunization. Genetic immunization was accomplished by biolistic bombardment using methods similar to those recently described[25]. Briefly, DNA-coated gold particles were prepared by combining 50 mg of 0.95 μm gold beads and 100 μl of 0.1 M spermidine and sonicating for 5 s. Plasmid DNA (100 μg) and $CaCl_2$ (200 μl) were added sequentially to the beads spinning in a vortex mixer. This mixture was allowed to precipitate at room temperature for 5–10 min. The bead preparation was then centrifuged (10,000 r.p.m. for 30 s) and washed 3 times in cold ethanol before resuspension in 7 ml of ethanol to give a final concentration of 7 mg gold per milliliter. The solution was then loaded into Tefzel tubing (Agracetus, Middleton, Wisconsin) and allowed to settle for 5 min. The ethanol was removed and the beads were attached to the sides of the tubing by rotation at 20 r.p.m. for 30 s and N_2 dried. The dry tubing lined with beads was then cut into 0.5-inch sections and stored for use with desiccant in parafilm-sealed vials. Animals were vaccinated by delivery of two shots (each shot consisted of 0.5 mg gold beads in 0.5 inch of tubing) to the shaved abdominal region using the *Accell* gene delivery device (Agracetus) at a discharge pres-

sure of 400 p.s.i. This delivers approximately 1.00 μg/DNA per shot. Animals were immunized with either the pAc-neo-OVA plasmid[21] or the plEglacZ plasmid (kindly provided by L. Huang), which contains the *lacZ* gene under the control of the CMV promoter. In some experiments, mice were immunized as described except that particles were coated with the pGREEN LANTERN-1 plasmid (Gibco BRL, Gaithersburg, Maryland), which contains the "humanized" reporter gene encoding GFP from the *Aequorea victoria* jellyfish. This gene encodes a naturally fluorescent protein requiring no substrates for visualization.

Cytotoxicity assays. Splenocytes from immunized animals were restimulated with minor modifications of previously described protocols[12]. Briefly, 1 week after immunization, splenocytes (30×10^6) were restimulated by coculture with irradiated (20,000 rad) EG7 cells (10×10^6). Effector cells were harvested 5 days later and cultured with 2×10^4 ^{51}Cr-labeled targets in round-bottom microwells (200 μl) at the indicated effector:target cell ratio. In some cases the effector cells were depleted of T-cell subsets using mAb plus complement before assay as described[22]. After 4 h at 37 °C, 100 μl supernatant from triplicate microcultures was collected and counted, and the percentage of specific release was calculated as described[12]. Results are reported as the mean of triplicate cultures. The s.e.m. of triplicate cultures was always less than 15% of the mean.

Protection assays. C57BL/6 mice were immunized as described with the indicated antigen–gene construct. Animals were challenged with tumors and evaluated for tumor survival as described[12]. Briefly, 7 days after the final immunization (day 0), OVA-immunized or *lacZ*-immunized animals were challenged by intradermal injection in the mid-flanks bilaterally with melanoma cells (2×10^4) at two times the dose lethal to 50% of the animals tested (LD_{50}). Survival is recorded as the percentage of surviving animals. Melanoma cells for injection were washed three times in PBS. Injected cells were greater than 95% viable by trypan blue exclusion. All experiments included five mice per group and were repeated at least three times. Mice that became moribund were killed according to animal care guidelines of the University of Pittsburgh Medical Center. In some experiments, animals were depleted of $CD8^+$ cells. This was accomplished by i.p. injection of CD8 mAb (2.43) 7 and 9 days after immunization as described, followed by tumor challenge on day 10 (ref. 12).

Electron microscopy. For electron microscopy, 24 h after immunization animals were killed, and draining lymph nodes were harvested and fixed in 2.5% glutaraldehyde for 1 h. Following fixation, nodes were washed in PBS, diced into 1-mm cubes, postfixed for 1 h in 1% aqueous osmium tetroxide, dehydrated through graded alcohols and embedded in Epon (Energy Beam Sciences, Waltham, Massachusetts). Following embedment, thin (60-nm) sections were cut using a Reichert Ultracut S (Leica, Chicago, Illinois) microtome, mounted on copper grids, counterstained with 2% uranyl acetate (7min) and 1% lead citrate (2 min), dried and observed using a JEOL 100CX microscope (JOEL, Chicago, Illinois). Morphologic indices were used to confirm the identity of the cell, principally these are size, a paucity of cytoplasmic granules, and the presence of cytoplasmic veils. To confirm the cytoplasmic locale of the gold particles, serial sections were cut (separated by 150 nm).

Fluorescence microscopy. Mice were immunized as described in 1 except that particles were coated with the pGREEN LANTERN-1 plasmid, which encodes GFP. Draining lymph nodes were excised 24 h after immunization.

Cytospins of lymph nodes were used to detect GFP-positive cells by fluorescence microscopy. Cells were centrifuged at 500 r.p.m. for 5 min, and mounted directly in an aqueous mounting medium (Gelvatol, Monsanto). Observation was with a Nikon FXL (Chicago, Illinois) photomicroscope using a 60X 1.4NA plan-apochromatic objective. All images were collected digitally using a Sony 3 chip color camera. In the case of fluorescence images, three individual frames were integrated to obtain an optimally contrasted image.

In order to detect the presence of skin-derived dendritic cells and the presence of GFP within these cells, lymph nodes from mice painted with rhodamine immediately before immunization were harvested, fixed in 2% paraformaldehyde in PBS, cryoprotected in 30% sucrose, and shock frozen. Cryosections (5 μm) were cut through the belly of the node, mounted on Superfrost slides (Fisher, Pittsburgh, Pennsylvania) and kept frozen until observation. Following removal from the cryostat chamber, sections were immediately mounted and observed, as described above. At low power, the skin-derived cells within the node are detected with a rhodamine cube set.

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